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The maternal-effect gene *futile cycle* is essential for pronuclear congression and mitotic spindle assembly in the zebrafish zygote

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SUMMARY

Embryos have been successfully used for the general study of the cell cycle. Although there are significant differences between the early embryonic and the somatic cell cycle in vertebrates, the existence of specialised factors that play a role during the early cell cycles has remained elusive. We analysed a lethal recessive maternal-effect mutant, *futile cycle* (*fue*), isolated in a maternal-effect screen for nuclear division defects in the zebrafish (*Danio rerio*). The pronuclei fail to congress in zygotes derived from homozygous *fue* mothers. In addition, a defect in the formation of chromosomal microtubules prevents mitotic spindle assembly and thus chromosome segregation in *fue* zygotes. However, centrosomal functions do not appear to be affected in *fue* embryos, suggesting this mutant blocks a subset of microtubule functions. Cleavage occurs normally for several divisions resulting in many anucleate cells, thus showing that nuclear- and cell division can be uncoupled genetically. Therefore, we propose that in mitotic spindle assembly chromosome-dependent microtubule nucleation is essential for the coupling of nuclear and cell division.

Key words: Maternal-effect mutants, Pronuclear congression, Mitotic spindle, Cell cycle, Zebrafish

INTRODUCTION

Maternal regulation is a key feature of early development with many components necessary for embryogenesis being deposited by the mother into the oocyte. In zebrafish, the genome is silenced until the ninth cleavage cycle (Kane and Kimmel, 1993), indicating that meiosis, mitosis and many other essential processes required for embryonic development are regulated by maternal factors until this stage.

Meiosis, the process by which haploid germ cells are formed, consists of two consecutive nuclear divisions in the absence of DNA replication (Nebreda and Ferby, 2000). During metazoan oogenesis, mature oocytes are arrested at specific stages of meiosis prior to fertilization. Oocytes from fish, amphibians and most mammals arrest at metaphase II of meiosis (Austin, 1965; Streisinger et al., 1981; Selman et al., 1993). Activation of the oocyte is in most species induced by sperm entry, resulting in a wave of cytosolic calcium at the moment of fertilization (Stricker, 1999) thereby causing a decrease in cyclin-dependent kinase activity, and inducing the oocyte to resume meiosis (Kline and Kline, 1992; Sensui and Morisawa, 1996; McDougall and Levasseur, 1998; Levasseur and McDougall, 2000). This leads to one set of sister chromatids being extruded to produce a haploid oocyte nucleus.

Following the completion of meiosis the haploid maternal- and paternal-derived genomes combine to create a diploid zygote. Studies in *Drosophila*, mouse and human show that the pronuclei migrate towards each other (congression) and subsequently enter mitosis (Schatten et al., 1985; Simerly et al., 1995; Callaini and Riparbelli, 1996). The mixing of the maternal and paternal chromosomes therefore occurs at the first mitosis. In species such as sea urchin, *Caenorhabditis*, *Xenopus*, and as demonstrated here the zebrafish, a diploid nucleus forms through the fusion of two haploid pronuclei before the first mitosis (Longo and Anderson, 1968; Ubbels et al., 1983; Strome and Wood, 1983).

Entry into mitosis is induced by the activation of maturation promoting factor (MPF), also referred to as cyclin-dependent kinase (Cdk1) (Evans et al., 1983; Murray and Kirschner, 1989; Murray et al., 1989). Active MPF triggers a cascade of events including nuclear envelope breakdown, chromosome condensation, mitotic spindle assembly and cyclin destruction by the anaphase promoting complex (APC). Cyclin destruction in turn results in MPF inactivation, thereby terminating mitosis (Murray and Hunt, 1993; Zachariae and Nasmyth, 1999).

Early embryonic, maternally regulated vertebrate cell cycles differ substantially from somatic cell cycles. Early embryonic cells have rapid synchronous cell cycles with alternating M (mitosis) and S (DNA synthesis) phases and lack G (gap) phases. The rapid repeated cleavages result in cells with successively smaller volumes. In many organisms the cleavages of early embryos appear to proceed irrespective of various perturbations (Nagano et al., 1981; Gerhart et al., 1984;

Newport and Kirschner, 1984; Gautier, 1987; Dabauvalle et al., 1988). Established techniques that interfere with meiosis or the first mitosis can be used to generate diploid gynogenic or androgenic zebrafish embryos (Streisinger et al., 1981; Corley-Smith et al., 1996), showing that severe disruption of these crucial processes does not lead to cell cycle arrest at early stages of zebrafish development.

One powerful approach to identify components that are specific for the early cell cycles is to perform a genetic screen for maternal-effect mutants. Such screens in *Drosophila* have already led to the isolation of several mutants that are specific for the early cell cycle (Glover, 1989; Foe et al., 1993). However, to date no such screens have been done in a vertebrate system. Although early cell cycles differ between *Drosophila* and vertebrates, it could be anticipated that specialised factors are also involved in the maternally regulated vertebrate cell cycle. Here we report the results of the first small scale maternal-effect screen for nuclear division defects specific for the early embryonic vertebrate cell cycle. We describe a lethal recessive maternal-effect mutation, *futile cycle* (*fue*), that abolishes pronuclear congression and chromosomal segregation during mitosis in all offspring of homozygous females. This mutation does not interfere with cytokinesis and thus permits several anucleate cleavage cycles. The homozygous mothers exhibit no defects other than the inviability of their progeny, suggesting the existence of a specific factor required for the early embryonic cell cycle in a vertebrate.

MATERIALS AND METHODS

Fish maintenance

Fish were raised as described by Mullins et al. (Mullins et al., 1994) and Haffter et al. (Haffter et al., 1996). All fish were bred in a Tübingen background. Maternal-effect mutants were generated in a gynogenesis-based maternal screen as previously described (Pelegri and Schulte-Merker, 1999). To maintain mutant lines, males from crosses containing identified homozygous mutant females were randomly outcrossed. Several incrosses were made from every outcross raised. Subsequently these incrosses were screened to recover the homozygous *fue* females. Maternal-effect embryos were obtained by mating homozygous mutant females with wild-type males or by in vitro fertilization using wild-type sperm.

In vitro fertilization

Males were anaesthetised in 0.2% ethyl-m-aminobenzoate metanesulphonate solution pH 7 (MESAB; Sigma) and then decapitated. The testes were dissected, and sheared in Hanks buffer (Westerfield, 1994; Pelegri and Schulte-Merker, 1999) to release sperm (6 testes per ml buffer). The sperm suspension was kept on ice allowing debris to settle prior to use. Oocytes were obtained from females by dry stripping. About 50 μl of sperm suspension was added to each clutch of eggs. After 1 minute, 1 ml E3 water (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM $MgSO_4$) was added, followed shortly thereafter by another 20 ml. All in vitro fertilizations and subsequent development occurred at 28°C.

Fluorescent labelling

Actin labelling was carried out on formaldehyde-fixed embryos that had been washed and permeabilised by incubation for 3 hours in phosphate-buffered saline (PBS) with 1% Triton X-100 (t-octylphenoxypolyethoxyethanol; Sigma). Actin filaments were labelled by incubation overnight at room temperature (RT) with 1:250 dilution of fluorescein phalloidin (Molecular Probes) in PBS containing 0.4% Triton X-100 (PBST). Nuclei were labelled using 4′,6-diamidino-2-phenyl indole (DAPI; 0.5 μg/ml) in PBST (Sigma). Embryos were incubated in DAPI solution for 10 minutes at RT followed by three washes in PBST of 5 minutes each.

Immunocytochemistry

In order to label microtubules, embryos were mechanically dechorionated and subsequently fixed in 4% formaldehyde, 0.05% glutaraldehyde, 5 μM EGTA, 5 μM $MgSO_4$ and 0.1% Triton X-100 in PBS for 1 hour at RT. One- and two-cell stage embryos were additionally treated with pronase (1 mg/ml for 2 minutes; Sigma) prior to fixation. For all other procedures embryos were fixed in 4% formaldehyde in PBS at 4°C overnight. The embryos were washed in PBST, mechanically dechorionated and then dehydrated through a dilution series into pure methanol at –20°C for at least 30 minutes. After rehydration the yolk was removed and the embryos were washed in PBST. Subsequently the embryos were blocked for at least 1 hour at RT in PBST with 1% bovine serum albumin (BSA; Sigma) (blocking buffer). Incubation with the primary antibody; mouse IgG anti-α-tubulin (Sigma; clone DM1α), mouse IgG anti-γ-tubulin (Sigma; clone GTU-88), mouse IgG anti-β-catenin (Sigma; clone 15B8), or mouse IgM anti-H5 (Hiss Diagnostics), diluted 1:1000 in blocking buffer took place overnight at 4°C. Then the embryos were washed in blocking buffer. Bound primary antibodies were detected using Cy5 or FITC-conjugated goat anti-mouse antibodies (both 1:300; Dianova), incubated overnight at 4°C. Following this incubation, the embryos were washed in PBST and transferred through a glycerol (Gerbu) dilution series into 70% glycerol. Polyvinyl alcohol (Mowiol 4-88; Hoechst) was used for mounting embryos and 1,4-diazobicyclooctane (DABCO; Merck) was added to the mounting medium to reduce fading of the samples. Images were obtained using a Leica confocal microscope, and digitally processed using Adobe PhotoShop software (Adobe Systems Inc.).

BrdU labelling

Bromodeoxyuridine (BrdU; ICN), dissolved in PBS at a concentration of 10 mM, was injected into embryos (5 nl each). After one cell cycle the embryos were fixed in 4% formaldehyde, 0.25% glutaraldehyde, and 0.1% Triton X-100 in PBS to preserve the structure and they were permeabilised as described. The embryos were then incubated in 4 M HCl for 20 minutes. Subsequently embryos were washed in PBST several times. Immunocytochemistry was performed according to standard protocols. After labelling with the primary mouse α-BrdU (ICN; clone II5B) antibody (1:50) followed by the secondary horse α-mouse alkaline phosphatase-conjugated (AP) antibody (Vector; 1:500) and washing in PBST, the embryos were rinsed three times in phosphatase buffer (100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl and 0.1% Tween 20). The enzymatic reaction was performed in phosphatase buffer with the substrates 4-nitro blue tetrazolium chloride (NBT; Sigma) and 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP; Gerbu). The embryos were rinsed in PBST and fixed after a visible precipitate had formed. Images were taken using a Zeiss Axiophot microscope.

For in vivo sperm labelling BrdU was used as a marker. To avoid diffusion of BrdU before the fish consumed the food and to be able to give a steady dose of label, we injected bud stage embryos with BrdU dissolved in PBS in order to feed these to the fish. Young males were fed 150 μg BrdU per gram body weight, on alternate days during a period of 50 days. The males were regularly mated during this period and fertilized eggs were collected and subsequently fixed at the two-cell stage in order to test for BrdU incorporation as described.

Drug treatment

The mycotoxin aphidicolin from *Nigrospora sphaerica* (Sigma) was dissolved in 50% dimethylsulfoxide (DMSO; Merck) at a concentration of 2.5 mg/ml, and diluted fivefold with PBS prior to

use. A volume of 35 nl was injected per embryo at approximately 20 minutes post-fertilization.

RESULTS

fue embryos undergo anucleate cleavages

We isolated the *fue* mutant as part of a small scale zebrafish maternal-effect screen (Pelegri and Schulte-Merker, 1999). We screened approximately 200 genomes and recovered 12 mutants of which 2 are involved in the process of nuclear division. Females were selected that gave rise to embryos that arrested cleaving after they had passed through several cell cycles and these were then rescreened for nuclear division defects. During cleavage the majority of *fue* embryos are morphologically indistinguishable from wild-type embryos. The cleavage orientation pattern – characteristic for the early wild-type embryo – is retained (Kimmel and Law, 1985; Kimmel et al., 1995), and the timing of these cleavages appears to be unaltered (Fig. 1A,B). However, after the embryos have undergone approximately ten cell cycles cleavage is arrested (Fig. 1C,D) and they eventually degrade. DNA labelling with DAPI at the early cell stages in *fue* embryos showed a total of two nuclei (and a polar body, which is often retained on the cell surface until the two-cell stage as in wild-type embryos). While cleavage proceeds normally in *fue* embryos, most cells go through several anucleate divisions.

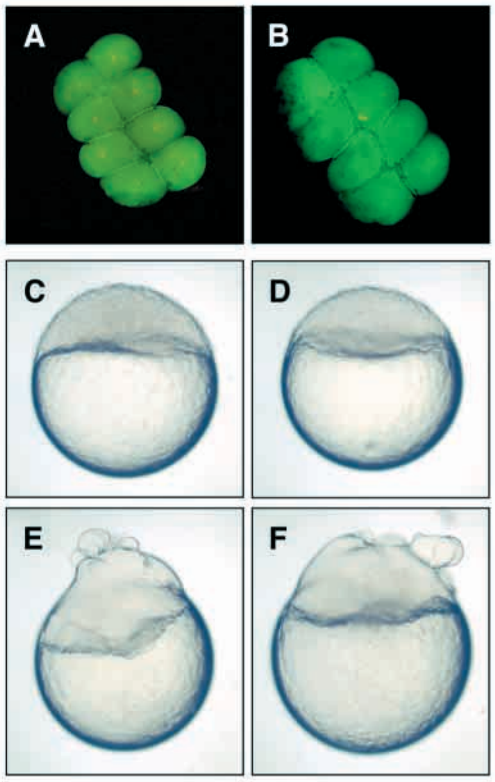

Fig. 1. *fue* embryos undergo several rounds of anucleate cleavage. Fixed wild-type (A) and *fue* (B) embryos at the eight-cell stage, labelled with DAPI to reveal nuclei (red) and fluorescein phalloidin to visualise the actin cytoskeleton (green). (C) A wild-type embryo at the sphere stage and (D) a living *fue* embryo shortly after it has arrested cleavage. In both cases development took place for the same period of time. In most of the *fue* embryos, the characteristic cleavage orientation pattern is not perturbed, and the cells progress through several cleavage cycles, indicating that the core cell cycle engine is not affected. (E,F) Unfertilized wild-type and *fue* eggs, showing that the sperm is required to initiate cleavage of the *fue* embryo as in the wild type.

Several distinct essential processes precede the first cleavage of the zebrafish zygote. Immediately after fertilization meiosis is resumed with the extrusion of the second polar body. This is followed by DNA replication, pronuclear congression/fusion and the segregation of the chromosomes during the first mitosis. Subsequently the embryo undergoes rapid cell cycles until it arrives at the mid blastula transition (MBT), when the silenced genome is activated and the rate of cleavage slows down. In the following part of this section we address which of these processes is affected in *fue* embryos.

Meiosis is completed normally in *fue* zygotes

At the end of oogenesis, the mature oocyte is arrested at metaphase of the second meiotic division in the zebrafish (Streisinger et al., 1981; Selman et al., 1993). Fertilization triggers the resumption of meiosis with the extrusion of a haploid set of chromosomes into a vesicle on the outside of the cell membrane, termed the second polar body. This event, which marks the completion of meiosis, occurs at about 7 minutes post-fertilization in wild-type zygotes (Streisinger et al., 1981). Since meiosis is the earliest process that could be affected in this mutant, we tested if the extrusion of the second polar body occurs normally in *fue* zygotes. In order to visualise the polar body, both DNA and actin were labelled. Extrusion of the second polar body in *fue* zygotes was indistinguishable from that of wild type, as shown in Fig. 2, indicating a normal completion of meiosis.

Congression of the pronuclei is arrested

In several organisms pronuclear fusion occurs after congression of the pronuclei is complete (Longo and Anderson, 1968; Ubbels et al., 1983; Strome and Wood, 1983). In others the pronuclei congress and subsequently enter mitosis, but do not fuse (Schatten et al., 1985; Simerly et al., 1995; Callaini and Riparbelli, 1996). As pronuclear behaviour had not been studied before in the zebrafish, we labelled DNA of in vitro fertilized zygotes at successive time points following fertilization, and demonstrated that pronuclear fusion does occur in the zebrafish zygote, starting at 14 minutes and ending around 20 minutes post-fertilization, but is often not completed until the onset of mitosis (Fig. 3A-C).

The two nuclei in *fue* embryos could be derived from either an arrest after chromosomal segregation has occurred at the first mitotic division or from a failure of pronuclear congression. To distinguish between these possibilities we labelled the DNA of *fue* zygotes fixed at 2 minute intervals between 10 and 30 minutes post-fertilization. Fusion of the pronuclei, which normally occurs between 14 and 20 minutes post-fertilization, was never observed (Fig. 3D-F). As a further test, we made the pronuclei distinguishable by using sperm DNA that was in vivo labelled with bromodeoxyuridine (BrdU). A male with BrdU-labelled sperm was mated to both wild-type and homozygous *fue* females and the collected eggs were fixed at the two-cell stage. In wild-type embryos both nuclei are labelled by this stage suggesting that fusion of the labelled sperm pronucleus with the unlabelled oocyte

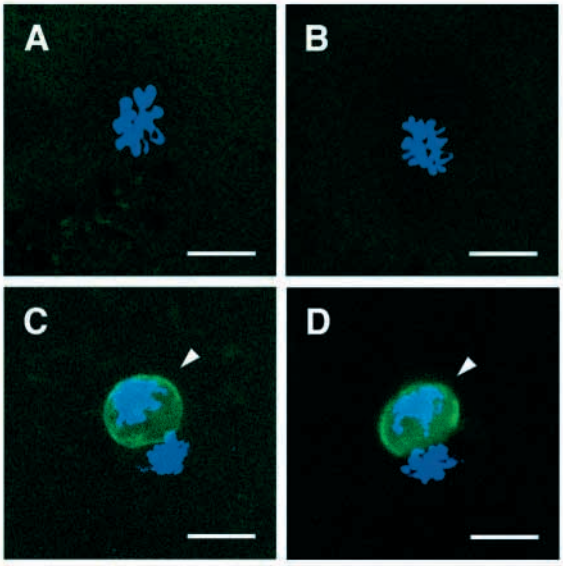

Fig. 2. *fue* zygotes resume and complete meiosis normally. DAPI was applied in order to label chromosomes (blue) and fluorescein phalloidin to visualise the actin cytoskeleton (green). (A,B) Chromosomes during meiotic arrest at metaphase II in wild-type (A) and *fue* (B) unfertilized, non-activated oocytes. Directly after fertilization meiosis is resumed and completed with the extrusion of one set of chromosomes into a vesicle on the outside of the cell membrane. Extrusion of this second polar body (arrowhead) takes place around 7 minutes post-fertilization in wild-type (C) and *fue* zygotes (D). This demonstrates that the terminal event of meiosis is completed in *fue* zygotes as in the wild type. Scale bars: 10 μm.

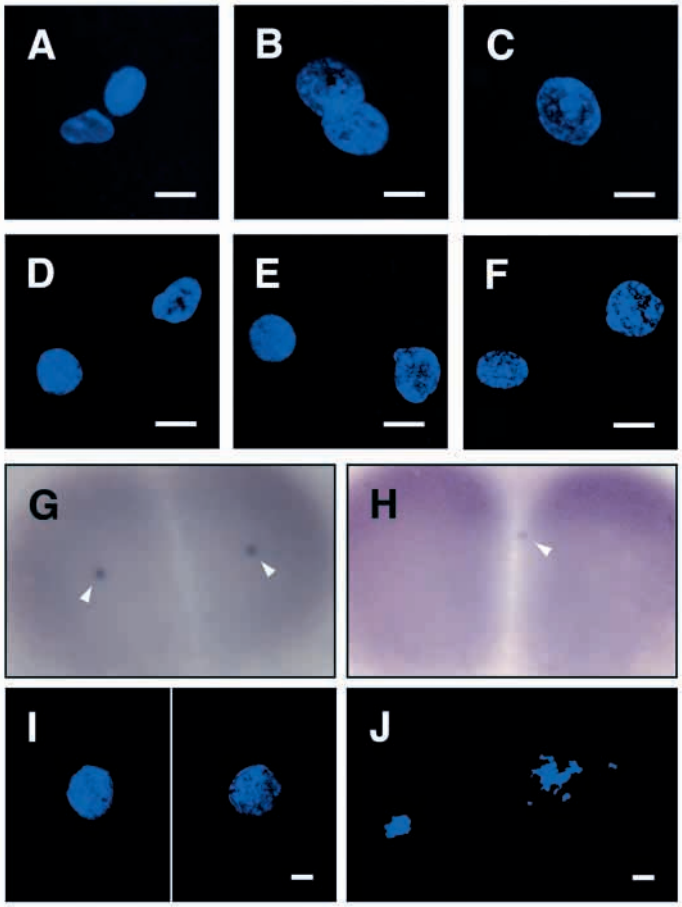

Fig. 3. The pronuclei fail to congress in *fue* zygotes. By labelling the nuclei of zygotes, fixed at consecutive time points after in vitro fertilization, we have documented the following sequence of pronuclear events. (A,D) Congression of pronuclei around 12 minutes post-fertilization, in wild-type (A) and *fue* (D) zygotes. (B,E) Around 14-20 minutes post-fertilization the pronuclei fuse in wild type (B) but are separate in *fue* zygotes (E). (C,F) Around 22 minutes post-fertilization one zygotic nucleus is present in wild type (C) and two pronuclei are observed in *fue* (F) zygotes. Eggs fertilized with in vivo BrdU-labelled sperm, were fixed, and nuclear BrdU was immunodetected at the two-cell stage. In wild-type embryos (G), two BrdU-labelled nuclei (arrowheads) are detected, in contrast to *fue* embryos (H) where only one BrdU-labelled nucleus (arrowhead) is detected, although two nuclei are always present in both *fue* (J) and wild-type embryos (I). This confirms that *fue* zygotes have a defect in pronuclear congression, and therefore fusion of the pronuclei does not occur as opposed to wild-type zygotes. Scale bars: 10 μm.

pronucleus occurred and that the newly formed zygotic nucleus then underwent mitosis thereby giving rise to two labelled nuclei (Fig. 3G). All *fue* embryos in which BrdU was detectable showed only one of the nuclei labelled (Fig. 3H), although two nuclei were always present at this stage (Fig. 3J). This indicates that the BrdU-labelled DNA of the male pronucleus never fuses with the female pronucleus. The nuclear BrdU label is specific since embryos obtained from crosses with males that did not possess BrdU-labelled sperm never showed labelled nuclei (not shown).

fue cells pass through consecutive S phases

Early embryonic cells progress through alternating cycles of S (DNA synthesis) and M (mitosis) phases and lack the G (gap) phases characteristic of somatic cell cycles (Murray and Hunt, 1993). We addressed whether both pronuclei in *fue* embryos undergo aspects of normal embryonic cell cycle, such as DNA synthesis. To investigate whether DNA replication occurs in *fue*, the embryos were assayed for the incorporation of BrdU into the DNA during S phase. BrdU was injected directly after the eggs were laid, and these embryos were fixed at the beginning of the two-cell stage. BrdU labelling of DNA was detected in both nuclei of *fue* embryos, indicating that DNA replication does indeed take place (Fig. 4A,B). To determine if replication continued during subsequent cell cycles we injected BrdU into embryos at the onset of the formation of the second furrow, when the DNA replication phase of the first two cycles has been completed, and fixed these embryos at the eight-cell stage. These *fue* embryos also showed nuclear BrdU labelling (Fig. 4C,D). We therefore conclude that DNA replication takes place in *fue* embryos at the one-cell stage and also during subsequent cell cycles.

Chromosomal segregation does not occur during mitosis

The segregation of chromosomes at mitosis occurs on an intricately constructed microtubule structure, the spindle. The first mitotic spindle is assembled in wild-type zygotes around 25 minutes post-fertilization (Fig. 5A) (Streisinger et al., 1981). During the first mitosis, two disorganised chromosome clusters are present in *fue* zygotes (Fig. 5B,C), alignment at the

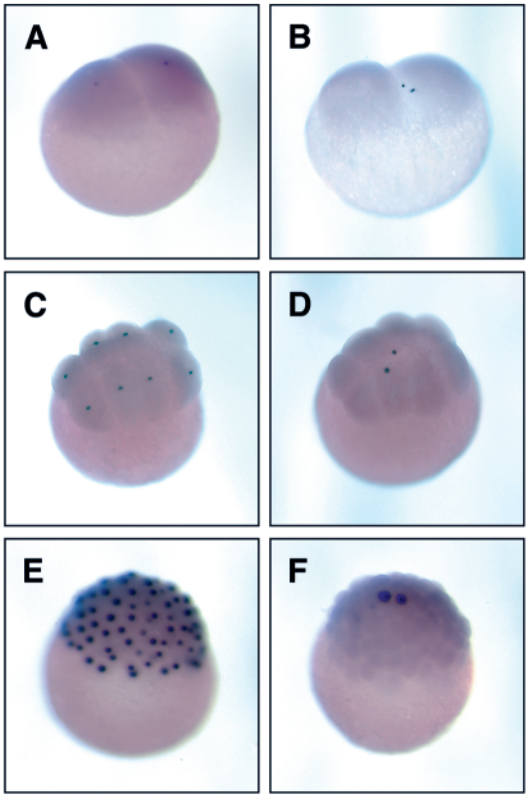

Fig. 4. DNA replication immunodetected after BrdU incorporation. (A,C,E) Wild type, (B,D,F) *fue* embryos. (A,B) Embryos injected with BrdU directly after fertilization show nuclear BrdU labelling at the beginning of the two-cell stage. (C,D) Embryos injected with BrdU at the onset of the second cleavage show nuclear BrdU labelling at the eight-cell stage. (E,F) When BrdU is injected at the 32-cell stage, we observed labelled nuclei at the 64-cell stage. These images illustrate that, *fue* zygotes undergo S phase and go through consecutive S phases during the subsequent cleavage cycles.

metaphase plate and segregation of chromosomes is never observed. When we labelled microtubules at the first mitosis, using an antibody directed against α-tubulin we observed two asters and occasionally nucleation of microtubules on one or both chromosome clusters (Fig. 5C). Therefore, in a number of zygotes the capacity of the chromosomes to induce microtubule nucleation is not lost. In these zygotes the microtubules are randomly distributed on one or both chromosome clusters (Fig. 5C), and are not organised into bundles or bipolar spindle-like structures. At subsequent mitoses aberrant spindles are observed in a minority of *fue* embryos and therefore chromosome clusters can be divided randomly (Fig. 5G). However, in most cases during mitosis at post-zygotic stages only microtubule nucleation on the chromosome clusters occurs (Fig. 5F).

The presence of asters at all mitoses in *fue* embryos suggests that microtubule nucleation by the centrosomes is not impaired. Further evidence that this process is normal comes from observing embryos treated with the mycotoxin

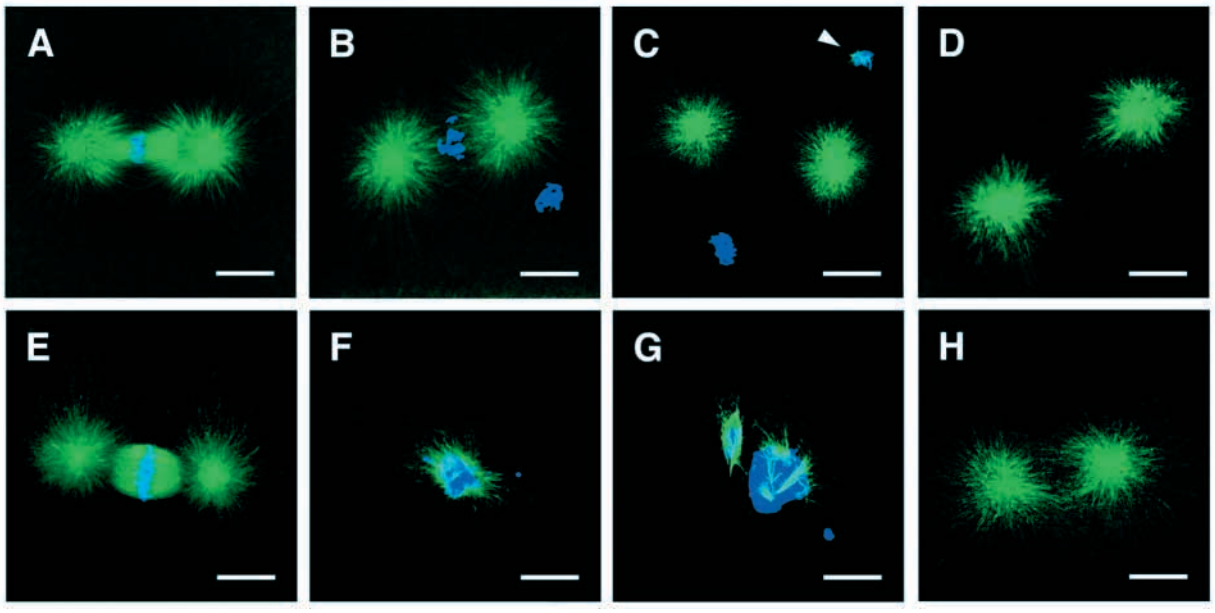

Fig. 5. *fue* mutants exhibit a defect in mitotic spindle assembly. Mitotic spindles were visualised using an antibody directed against α-tubulin (green) and chromosomes were labelled with DAPI (blue). (A) The first mitotic spindle is assembled in wild-type zygotes at 25 minutes post-fertilization. (B,C) In *fue* zygotes mitotic spindle assembly is perturbed because of a defect in chromosome-dependent microtubule nucleation (B), however, in about 30% of *fue* zygotes microtubule nucleation on one or both chromosome clusters (arrowhead, C) does occur. In all cases the formation of asters does not seem to be affected (B,C). (E) A mitotic spindle as observed in wild-type eight-cell stage embryos. (F,G) In the majority of *fue* embryos at post-zygotic stages, microtubules are associated with the chromosome clusters during mitosis (F), except in a minority of embryos (about 5%), where aberrant spindles are observed, which can randomly segregate DNA (G). (D,H) In all *fue* cells at post-zygotic stages a pair of asters is formed during mitosis (H), which is similar to that observed in the anucleate cells of wild-type embryos that were injected with the DNA replication inhibitor, aphidicolin (D). Therefore, *fue* zygotes demonstrate a defect in spindle assembly, arising from a defect in the chromatin-dependent generation of microtubules (Karsenti and Vernos, 2001). Scale bars: 20 μm.

aphidicolin, which has previously been shown to specifically inhibit DNA replication by interacting with DNA polymerase α (Ikegami et al., 1978). Injection of aphidicolin into early wild-type embryos induces anucleate, but otherwise normally cleaving cells. Phenocopying this aspect of *fue* by perturbing a different process allows us to compare the asters formed during mitosis. The asters observed in *fue* and aphidicolin-injected embryos are indistinguishable (Fig. 5D,H), indicating that the defect in microtubule nucleation in *fue* zygotes is restricted to the contribution of the chromosomes to mitotic spindle assembly.

We also tested the integrity of the centrosomes in *fue* cells by comparing these organelles in aphidicolin-injected embryos. Centrosomes were detected using an antibody directed against γ-tubulin, a highly conserved component of this organelle (Oakley et al., 1990; Stearns et al., 1991; Stearns and Kirschner, 1994). A pair of centrosomes is present in all cells at all stages of both *fue* and aphidicolin-injected embryos (Fig. 6A-C), showing that centrosome duplication is not affected. In addition, the presence of a pair of asters at the first mitosis in *fue* embryos (Fig. 5B,C) indicates normal centrosomal duplication prior to this event. Centrosomes of *fue* and aphidicolin-treated embryos are indistinguishable from those of wild type (Fig. 6D-F). Since these organelles are crucial for cytokinesis their presence and capacity to duplicate could be expected.

The presence of two asters, but the absence of a mitotic spindle indicates that chromosomal microtubules required for spindle assembly cannot form in most *fue* zygotes (Karsenti and Vernos, 2001). The absence of chromosomal segregation at the first mitosis suggests a role for *fue* in mitotic spindle assembly in addition to its earlier function in pronuclear congression.

Transcription is initiated earlier in *fue* embryos

During the mid blastula transition (MBT) the previously silenced genome becomes active in wild-type embryos, triggered by the decreased cytoplasm to DNA ratio (Newport and Kirschner, 1982; Edgar et al., 1986; Kane and Kimmel, 1993). As both cleavage and DNA replication proceed in *fue* embryos but chromosomal segregation does not take place, the DNA concentration increases locally more rapidly than normal as the cell size decreases. This should lead to an earlier arrival at the critical cytoplasm to DNA ratio in *fue* embryos than in wild-type embryos, and therefore activation of the *fue* genome is expected to occur at an earlier stage if transcription is not affected.

The phosphorylation of the H5 epitope of RNA polymerase II has been shown to coincide with the transcriptional activation of the silenced genome at MBT in several species (Seydoux and Dunn, 1997; Knaut et al., 2000). Therefore, we analysed *fue* embryos at several early cell stages using an antibody that detects the phosphorylation of this epitope (Bregman et al., 1995) as a marker for the onset of transcription.

We first observe phosphorylation of the H5 epitope of RNA polymerase II in wild-type embryos at the 512-cell stage and in *fue* embryos at the 32-cell stage (Fig. 7). The phosphorylation of the H5 epitope of RNA polymerase II in *fue* embryos suggests mRNA transcription does occur in this maternal-effect mutant. When we assume that the DNA concentration doubles with every cell cycle and cleavage proceeds normally in *fue* embryos one would predict transcriptional activation in these embryos should occur after half the number of cell cycles that it takes wild-type embryos to reach MBT, or five cycles (32-cell stage) instead of nine cycles (512-cell stage). Therefore the earlier initiation of transcription in *fue* embryos seems to provide genetic support for transcriptional initiation being regulated by a previously proposed mechanism based on the DNA to cytoplasm ratio.

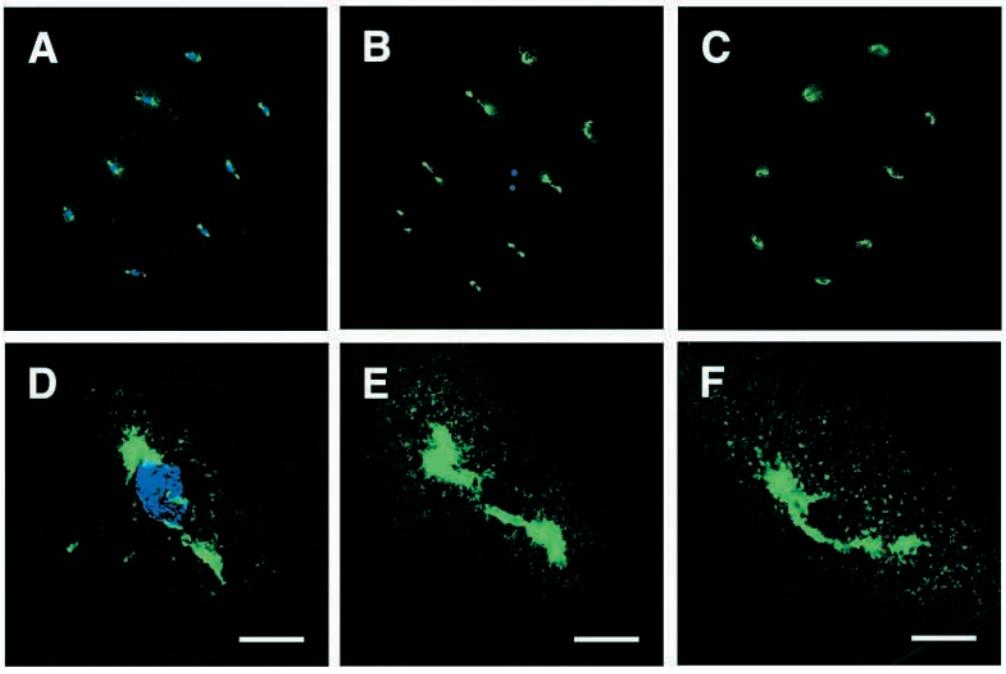

Fig. 6. Centrosome duplication is not affected in *fue* mutants. Centrosomes at the eight-cell stage during interphase, visualised with an antibody directed against γ-tubulin (green) and nuclei labelled with DAPI (blue) from (A) a wild-type embryo, (B) *fue* embryo, (C) an embryo treated with the DNA replication inhibitor aphidicolin. (D-F) Enlargements of single centrosome pairs of A-C, respectively. Centrosomes are present and are able to duplicate in all *fue* cells as well as the (anucleate) cells of aphidicolin-treated embryos, demonstrating that the centrosomes can duplicate independently from nuclei. Scale bars: 20 μm.

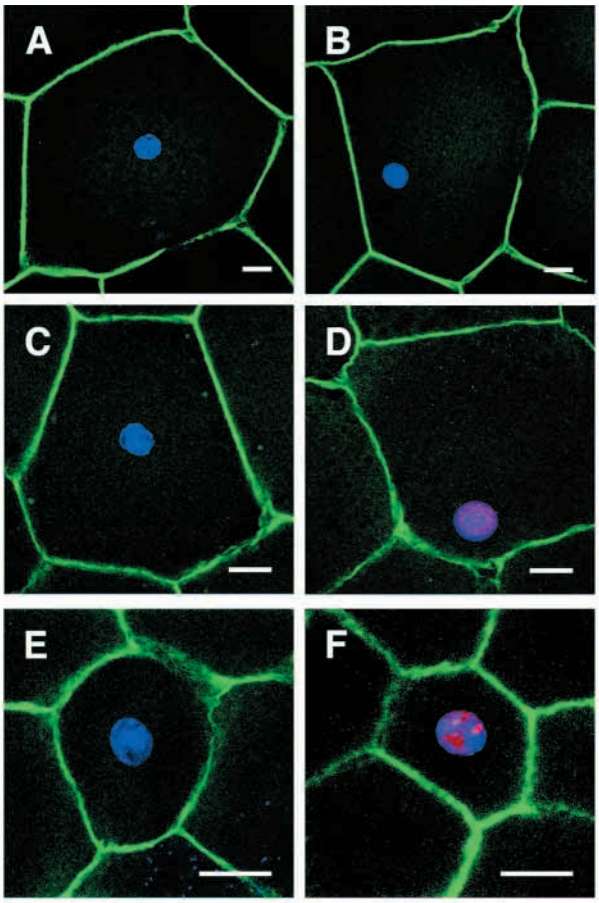

Fig. 7. Transcription is initiated earlier in *fue* embryos. Initiation of mRNA transcription coincides with phosphorylation of the H5 epitope of RNA polymerase II. Labelling with an antibody directed against the phosphorylated form of this epitope is in red, nuclei are labelled with DAPI (blue) while the outline of the cell is visualised with an antibody directed against β-catenin (green). (A,C) Wild-type and (B,D) *fue* cells from (A,B) 16-cell stage and (C,D) 32-cell stage embryos. (E,F) Wild-type embryos at the 256-cell stage (E) and 512-cell stage (F). Phosphorylation of the H5 epitope occurs first at the 512-cell stage in wild-type embryos and at the 32-cell stage in *fue* embryos, showing that the onset of mRNA transcription occurs at an earlier stage in *fue* embryos. Scale bars: 20 μm.

DISCUSSION

The free running cell cycle oscillator

Although cell division has been studied for over a century, the existence of specific factors that may play a role during the first embryonic cell cycles has not been demonstrated in a vertebrate, despite these early divisions possessing several features uncommon to the later occurring somatic cell cycles. We present an analysis of a maternal-effect mutant, *futile cycle* (*fue*), that has severe defects in several processes at the first embryonic cell cycles. In zygotes derived from homozygous *fue* females the pronuclei do not congress and therefore never fuse. However, *fue* zygotes enter mitosis without delay. In the majority of *fue* zygotes spindle assembly is perturbed at the first mitosis because of a defect in chromosome-dependent microtubule nucleation, and therefore chromosomal segregation is abolished. Following mitosis, *fue* zygotes proceed to cleave as in the wild type, thereby genetically uncoupling nuclear division from cell division. Incorporation of the thymidine analogue BrdU into the DNA indicates that *fue* embryos pass through consecutive S phases. Since *fue* embryos undergo normal cleavages, this results in a local accumulation of DNA in two clusters within one or two cells, with the remainder of the cells being anucleate. As the vast majority of cells are anucleate their cleavage must be driven by maternally supplied factors. The eventual arrest of cleaving after *fue* embryos have passed through several cell cycles is likely to be due to the depletion of these maternal products.

Cyclical oscillations in the activity of maturation promoting factor (MPF) have been shown to constitute the core of the cell cycle oscillator, determining when the cell enters mitosis and when it exits to enter interphase (Evans et al., 1983; Murray and Kirschner, 1989; Murray et al., 1989). Active MPF induces the downstream events of mitosis, while inactive, it permits downstream events that lead to interphase. In *fue* embryos, cleavage takes place as in wild-type embryos, indicating that the abolished factor is not involved in the periodical change of MPF activity but is an event occurring downstream of this oscillating mechanism.

Pronuclear congression is abolished in *fue* zygotes

Pronuclear fusion follows the completion of meiosis in several organisms (Longo and Anderson, 1968; Ubbels et al., 1983; Strome and Wood, 1983), however, this had not been demonstrated in zebrafish. By visualising the nuclei of zygotes that were fixed at consecutive time points after in vitro fertilization, we have been able to document a sequence of pronuclear events that takes place in the zebrafish zygote. We show that pronuclear fusion occurs in wild-type zebrafish zygotes between 14 and 20 minutes post-fertilization. In contrast, we did not observe pronuclear fusion in *fue* zygotes. Furthermore, to distinguish between the pronuclei, we labelled sperm DNA in vivo using BrdU as a marker. Following fertilization we determined the number of BrdU-labelled nuclei shortly after the first mitosis was completed. Only one BrdU-labelled nucleus could be detected in two-cell *fue* embryos, although two nuclei are always present at this stage, confirming that pronuclear fusion does not occur in *fue* zygotes. This phenotype suggests a role for *fue* in pronuclear congression as the pronuclei are always observed at a distance from each other.

Mitotic spindle assembly is perturbed in *fue* zygotes

fue mutants demonstrate abnormal mitosis, suggesting that the *fue* wild-type gene product may also be required for chromosome segregation. Alternatively the mitotic defect may solely be a secondary consequence of the perturbed pronuclear congression. However, preventing pronuclear fusion using different approaches in several species has been shown not to block the zygote from entering mitosis or to prevent normal chromosomal segregation (Subtelny, 1958; H. Schatten, 1994; Sluder et al., 1995; Sadler and Shakes, 2000). In addition, *fue* zygotes enter mitosis with the appearance of asters and often microtubule nucleation on the chromosome clusters is observed at that time, which is not expected if such a checkpoint exists. Therefore it would seem unlikely that pronuclear fusion is a prerequisite for mitosis in zebrafish

zygotes, suggesting that the *fue* wild-type gene product may also be required at this stage.

During the first mitosis two chromosome clusters are present in *fue* zygotes, which often, but not always, have a defect in microtubule nucleation. When microtubules are present on the chromosomes they do not organise into bundles or bipolar spindles, suggesting a defect in coalescence. The formation of normal asters shows that centrosome-dependent organisation of microtubules is not affected in *fue* zygotes, indicating that the defect is restricted to the chromosome-dependent nucleation of microtubules. At later stages chromosome clusters mostly nucleate microtubules and with a low incidence can even randomly segregate DNA. Therefore, the defect at post-zygotic mitoses is less severe than at the first mitosis, which may be due to a greater amount of DNA present in these clusters.

Since chromosome clusters are observed during mitosis, *fue* embryos do not have an obvious defect in chromosomal condensation. Blow and Watson have demonstrated a requirement of the nuclear envelope for DNA replication (Blow and Watson, 1987), therefore the ability of *fue* embryos to replicate the chromosomes could indicate the presence of a functional nuclear envelope. In addition, this envelope has to break down or enter a permeable state in order to allow the subsequent round of DNA synthesis (Blow and Laskey, 1988; Coverley et al., 1993). Furthermore, the presence of microtubules on some chromosome clusters indicates the absence of a nuclear envelope at mitosis, therefore suggesting nuclear envelope break down does occur. The kinetochores play a role in the stabilisation of microtubules as well as positioning of chromosomes at the metaphase plate. Since all sperm-derived components in *fue* embryos are wild type, the paternal chromosomes are expected to posses normal kinetochores. However, a significant difference is not observed between the paternal and maternal derived chromosome clusters at mitosis, suggesting that the defect in *fue* is not related to kinetochore function. Although, as not all kinetochore-associated proteins may be provided by the sperm, we cannot completely exclude crucial components of kinetochores from being affected.

Nucleation of microtubules at the chromosomes has previously been shown to be induced by the GTPase Ran (Wilde and Zheng, 1999; Carazo-Salas et al., 1999; Carazo-Salas et al., 2001), which is present as a gradient with the highest concentration of the GTP-bound form on the chromosomes. Several proteins essential for mitotic spindle assembly are inactivated by being bound to importin β and released around the chromosomes during mitosis by RanGTP (Gruss et al., 2001; Nachury et al., 2001), thereby inducing spindle assembly. We have shown that *fue* could block the pathway that leads to the chromatin-dependent generation of microtubules, which is regulated by Ran, its GTP exchange factor, and its downstream components (Karsenti and Vernos, 2001).

fue couples karyo- to cytokinesis

Pronuclear congression is a microtubule-mediated process, in which the centriole provided by the sperm assembles an aster that connects it to the oocyte nucleus, thereby bringing both pronuclei together (Lessman and Huver, 1981; G. Schatten, 1994). The asters formed at mitosis in *fue* embryos are normal, suggesting that the sperm aster is normal too, and that the defect may be in the following step of bringing the pronuclei towards each other. Since the defect observed during mitosis is due to the generation or organisation of microtubules, both defects in *fue* zygotes appear to primarily affect microtubule function. Interestingly, directly after fertilization a polar body is extruded in *fue* zygotes, showing that meiosis is completed successfully and thus indicating that the meiotic spindle is normal. Therefore, the *fue* phenotype reveals a crucial difference between the processes of assembly of meiotic and mitotic spindles. Since *fue* embryos initiate cleavage as in wild-type embryos, centrosomal behaviour is not affected by the absence of pronuclear fusion and the improper mitosis. We confirmed the presence of centrosomes at all cell stages in *fue* embryos, consistent with their ability to duplicate being an absolute requirement for cell division (Rieder et al., 2001). Therefore, by specifically perturbing the contribution of the chromosomes in mitotic spindle assembly, while not affecting centrosomal functions, we propose a mechanism by which nuclear- and cell division are connected.

Like *fue*, the *Drosophila* maternal-effect mutant *giant nuclei* (*gnu*) has two nuclei per embryo as well as DNA replication in the absence of chromosomal segregation and normal centrosome duplication (Freeman et al., 1986). However, there are several phenotypic differences when compared with *fue* mutants. *gnu* embryos do not show a proper completion of meiosis as DNA replication is initiated before fertilization has occurred (Freeman and Glover, 1987). In addition, *gnu* embryos do not form pole cells. These cells give rise to the future germ cells and are the first to be formed in the *Drosophila* embryo, and therefore this maternal-effect mutation, unlike *fue*, does not uncouple karyokinesis from cytokinesis. It has been demonstrated that these processes can indeed be uncoupled in *Drosophila*, since embryos injected with the DNA replication inhibitor aphidicolin are anucleate, but still form pole cells (Raff and Glover, 1989). The *Drosophila* embryo is initially a syncitium, and therefore pole cell formation might be a mechanistically different process from cleavage in the vertebrate embryo. However, the differences observed between the *Drosophila gnu* and the zebrafish *fue* phenotype make it unlikely that this factor could also be affected in *fue* embryos.

Although we believe that the function of *fue* is restricted to the first embryonic cell cycles it could instead represent a hypomorphic allele that also acts zygotically like the *Drosophila* mutation *polo* (Sunkel and Glover, 1988; Fenton and Glover, 1993; Glover et al., 1998) which was discovered on the basis of a maternal phenotype. Alternatively, *fue* function could be redundant during the subsequent somatic cell cycles. Thus, *fue* may be revealed as a factor that also acts during the later somatic cell cycles. However, the maternal-effect mutation in *fue* causes lethality in all offspring at an early stage while the homozygous mothers appear healthy and do not exhibit either a shorter life span or decreased fecundity. Importantly, *fue* is the first mutant documented to have cell division in the absence of nuclear division, and thus represents a unique phenotype. Therefore, the characterisation of the *fue* gene product should provide important new insight into the processes of pronuclear congression and mitotic spindle assembly at the earliest stage of vertebrate development.

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